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SUPPRESSION OF P450IIC12 GENE EXPRESSION AND ELEVATION OF ACTIN MESSENGER RIBONUCLEIC ACID LEVELS IN THE LIVERS OF FEMALE RATS AFTER INJECTION OF THE INTERFERON INDUCER POLY rI·POLY rC

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Abstract—Interferons and interferon inducers cause a reduction in hepatic microsomal cytochrome P450 [AH, reduced-flavoprotein:oxygen oxidoreductase (RH hydroxylating), EC 1.14.14.1] content and drug-metabolizing activities in experimental animals. In the present study, the acute effects of administration of the interferon inducer polyriboinosinic acid·polyribocytidylic acid (poly rI·poly rC) to female rats on expression of the microsomal apoprotein and hepatic mRNA for P450IIC12, a constitutive enzyme comprising a significant fraction of the total P450 in untreated female rats, were examined. Poly rI·poly rC treatment (10 mg/kg, i.p.) caused a suppression of P450IIC12 apoprotein that was of greatest magnitude (47% of control levels) 24 hr after injection. P450IIC12 was still suppressed significantly ($P < 0.05$) 48 hr after treatment. The time courses of suppression and recovery of P450IIC12 protein, as well as the magnitude of the effect, were similar to those of total microsomal P450 measured spectrophotometrically. P450IIC12 mRNA levels were also suppressed by the poly rI·poly rC treatment, reaching 29% of control values within 24 hr. Comparison of the kinetics of suppression of the P450IIC12 mRNA and apoprotein indicated that at least part of the suppression of the protein is mediated pretranslationally. However, the existence of a posttranslational component could not be excluded. Concomitant with the suppression of P450IIC12, actin mRNA content was found to be elevated by at least 3.6-fold in the livers of poly rI·poly rC-treated female rats, with the maximum effect occurring 12 hr after injection of the drug. This effect of poly rI·poly rC on expression of actin mRNA appeared to be at least partially sex-specific, since in a previous study [Morgan ET and Norman CA, *Drug Metab Dispos* **18**: 649–653, 1990] a significant effect of the interferon inducer on actin expression was not observed in livers of male rats.

The ability of interferons, and compounds that induce the production of interferons, to suppress hepatic drug metabolism and to decrease hepatic levels of cytochrome P450 is well established [for a review, see Ref. 1]. Early studies on the mechanism of interferon-evoked suppression of P450 indicated that the decrease in P450 is accompanied by a decrease in a population of hepatic hemoproteins with a short half-life [2]. Electrophoretic analysis of microsomes from interferon-treated rats indicated a decrease in a population of proteins with mobilities in the P450 molecular weight range [3].

More recently, it has been shown that although total hepatic poly $(A)^+$ RNA is increased after treatment of rats with interferon inducers, its translation in rabbit reticulocyte lysate is inhibited [4]. In contrast, the mRNA for P450IVA1 is suppressed rapidly following the administration of interferon inducers to clofibrate-treated rats, showing that the mechanism for suppression of this inducible isozyme is pretranslational [1]. However, the latter study did not address the suppression of constitutive P450 gene expression by interferon inducers. On the other hand, experiments in our laboratory have shown that the major constitutive P450 isozyme in male rat liver, P450IIC11, is pretranslationally suppressed by interferon inducers, and that suppression of this isozyme contributes significantly to the overall decrease in hepatic microsomal P450 [5]. Pretranslational suppression of another male-specific rat liver isozyme, P450IIIA2, by recombinant interferon-γ has also been reported recently [6].

The purpose of the present study was to determine whether P450IIC12, a female-specific isozyme constituting about 25–50% [7] of the total P450 in female rat liver microsomes, is suppressed by interferon inducers, and if so, by what mechanism. The experiments demonstrated that although both P450IIC12 mRNA and apoprotein were suppressed by treatment of rats with polyribosinic acid·polyribocytidylic acid (poly rI·poly rC), there was no clear temporal distinction of these effects, indicating that both pre- and posttranslational components may contribute to the suppression. In addition actin mRNA, often used as a constitutively expressed control in mRNA regulation studies, was elevated 3- to 7-fold in the livers of female rats receiving poly rI·poly rC treatment.

MATERIALS AND METHODS

Animals and treatments. Female Sprague–Dawley rats, 7 to 10 weeks old, from Harlan Sprague–Dawley Inc. were used. They were allowed free access to food and water at all times. Poly rI·poly rC (Sigma) was dissolved in sterile saline (5 mg/mL)

and injected i.p. at a dose of 10 mg/kg. Animals were killed by CO_2 asphyxiation at the times indicated. Control animals received an equal volume of sterile saline and were killed 24 hr later.

Preparation of hepatic microsomes, total RNA and poly (A)$^+$ RNA. Livers were excised and perfused with cold 1.15% KCl. Pyrophosphate-washed microsomes were prepared as described previously [8], and stored at −80°. Total RNA was prepared from portions of the same livers, according to Chomczynski and Sacchi [9], and stored at −80°. RNA contents of the samples were estimated from their absorbances at 260 nm [10]. Further validation of these estimates was done by running equal amounts of each sample on an agarose gel in the presence of formaldehyde [10], staining with ethidium bromide, and visual inspection. Poly (A)$^+$ RNA was purified from total RNA by batch absorption on oligo(dT)-cellulose (Type II, Collaborative Research Inc., Bedford, MA).

Assays of microsomal proteins. Total microsomal protein was determined by the method of Lowry *et al.* [11]. Cytochrome b_5 content was assayed from the difference spectrum of NADH-reduced and oxidized microsomes at 424 nm [12], using an extinction coefficient of 112 mM^{-1}. Total microsomal P450 concentrations were determined from the CO difference spectrum of the reduced protein [12].

Western blot immunoassays. Relative levels of P450IIC12 in microsomal samples were measured by a previously described method [13]. Briefly, microsomal proteins were separated by polyacrylamide gel electrophoresis (7.5% polyacrylamide) in the presence of sodium dodecyl sulfate and blotted electrophoretically on nitrocellulose filters. The filters were blocked with buffer containing 1% bovine serum albumin, and probed with specific monoclonal antibodies to PC450IIC12. The antigen–antibody complexes were visualized using an appropriate horseradish peroxidase-labeled second antibody (Jackson Immunoresearch Laboratories, Inc.) with 4-chloro-1-naphthol as substrate. The intensities of the stained bands were measured using an LKB Ultroscan Laser Densitometer. Care was taken to ensure that signals generated were within the range given a response proportional to the amount of microsomal protein applied to the gel.

RNA slot blot assays. Relative levels of P450IIC12 in hepatic RNA samples were quantitated using the cloned cDNA C-6 as described previously [13]. Total RNA or poly (A)$^+$ RNA was denatured with formaldehyde, and three different amounts of each sample were applied to a nylon 66 filter in the wells of a slot-blot apparatus. After immobilizing the RNA by UV-irradiation, the filters were probed with the ^{32}P-labeled cDNA (Multiprime, Amersham) fragment and washed under conditions shown to produce specific detection of P450IIC12 mRNA [14]. The filters were then subjected to autoradiography, and the intensities of the signals quantified by densitometry. Again, care was taken to ensure that the signals generated were in the range giving a response proportional to the amount of RNA applied to the filter. Relative levels of β-actin mRNA in the samples were determined on duplicate blots by the same method, using a full-length β-actin cDNA probe [15].

Relative contents of poly (A)$^+$ RNA in the purified mRNA samples used for the above slot blots were measured by probing identical blots with 5′-^{32}P-labeled oligo-d$(T)_{16}$. For each slot blot containing 50–72 RNA samples, 20 pmol of 5′-hydroxy-d$(T)_{16}$ (Pharmacia LKB Biotechnology Inc., Piscataway, NJ) was labeled with polynucleotide kinase, and hybridized to the immobilized RNA at a concentration of 1 pmol/mL for 2 hr in 6 × SSPE (60 mM sodium phosphate buffer, pH 7.7, containing 1.08 M NaCl and 6 mM EDTA) at room temperature. The blots were washed twice with 2 × SSC (30 mM sodium citrate buffer, pH 7.0, 0.3 M NaCl) containing 0.1% sodium dodecyl sulfate for 5 min at room temperature, dried and subjected to autoradiography and densitometry as described above.

Statistical analysis. One-way analysis of variance and Dunnett's test were used to determine if a significant difference existed between the mean of each treatment group and the mean of the control group. Results were tested for equivalence of variance by Bartlett's test, and where appropriate the data were retested after logarithmic transformation. All results are expressed as the mean ± SEM, and N represents determinations on different animals within a group.

RESULTS

Elevation of actin mRNA by poly rI·poly rC treatment. Measurement of relative levels of a specific mRNA in total RNA samples using the RNA slot blot technique necessitates the use of a control to measure the amount of RNA actually applied to the filter. This is because total RNA isolated from tissues may have various degrees of contamination with proteins [10] and other molecules [10, 16] that may contribute to the absorbance at 260 nm which is used to estimate the RNA concentrations in the samples. As a gene product that is fairly abundant and constitutively expressed in all mammalian cells, actin mRNA is used frequently as the required "loading control" in Northern and RNA slot blots. Indeed, I have used this control in studies on regulation of P450IIC11 by bacterial endotoxin [13] and interferon inducers [5].

In the present study, when total RNA samples from the livers of poly rI·poly rC-treated female rats were used, actin mRNA levels were observed to be elevated more than 7-fold compared with livers from untreated animals (Fig. 1). To determine that this increase truly represented an increase in hepatic actin mRNA, poly (A)$^+$ RNA from each total RNA sample was purified, and the analysis repeated. The purified samples were analyzed for their contents of poly (A)$^+$ RNA by probing the blots with an oligo d$(T)_{16}$ probe. No significant differences were observed among groups in the yield of mRNA from total RNA samples (not shown). As shown in Figs. 1 and 2, treatment of the rats with poly rI·poly rC caused an elevation in hepatic levels of actin mRNA to 310% of control values within 6 hr. The maximum elevation of 360% was seen 12 hr after treatment, and this was followed by a decline to 218% of control

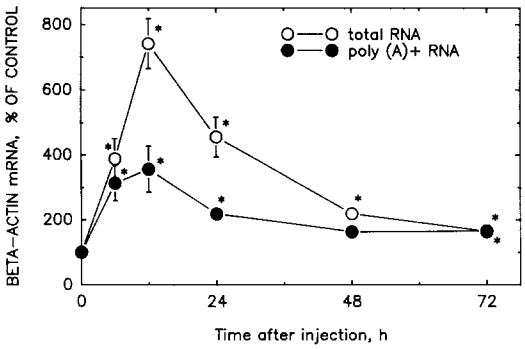

Fig. 1. Time course of the poly rI·poly rC-evoked elevation of actin mRNA in livers of female rats. Adult female rats were administered poly rI·poly rC (10 mg/kg) or saline and killed at the times shown. Relative hepatic levels of actin mRNA were determined by slot blotting of total hepatic RNA or poly $(A)^+$ RNA as described in the text. The relative contents of poly $(A)^+$ in the affinity purified samples were determined by probing the blots with an oligo-$d(T)_{16}$ probe as described. Values from the total RNA slot blots were uncorrected. The number of animals in each group was as follows: 0 (control), 8; 6 hr, 4; 12 hr, 5; 24 hr, 9; 48 hr, 6; and 72 hr, 4. Key: (*) significantly different from control value, $P < 0.05$. This figure summarizes data from the animals used in experiments 1 and 2, Table 1. The absence of an error bar means that the standard error for a given point is smaller than the size of the symbol.

values at 24 hr. Thereafter, actin mRNA levels declined to 165% of controls at 48 hr, and did not decline further by 72 hr.

In view of these results, subsequent studies on the regulation of the P450IIC12 mRNA by poly rI·poly rC were performed using purified poly $(A)^+$ RNA, with correction for mRNA content using the oligo-dT hybridization assay.

Suppression of P450IIC12 expression by poly rI·poly rC treatment. Treatment of female rats with poly rI·poly rC caused a decrease in the microsomal content of spectrally measurable P450, as described previously in male rats, hamsters and mice [1, 2, 5, 6, 17, 18], with the earliest significant effect occurring 12 hr after injection (Table 1). The largest decrease of 49% occurred 24 hr after treatment. Total P450 levels had begun to return to normal at 48 hr, although they were still significantly depressed at 72 hr. Microsomal cytochrome b_5 content was also lowered by poly rI·poly rC treatment, although no significant effect was seen until 24 hr after injection of the drug (Table 1).

Microsomal levels of P450IIC12 apoprotein were suppressed significantly ($P < 0.05$) at 12, 24 and 48 hr after poly rI·poly rC injection, with the lowest level of expression (47% of control levels) occurring at 24 hr (Figs. 3 and 4). The P450IIC12 content of the microsomes was no longer significantly different from control by 72 hr after treatment. Hepatic levels of the P450IIC12 mRNA were also suppressed to 33 and 29% of control values at 12 and 24 hr, respectively, after treatment with the interferon

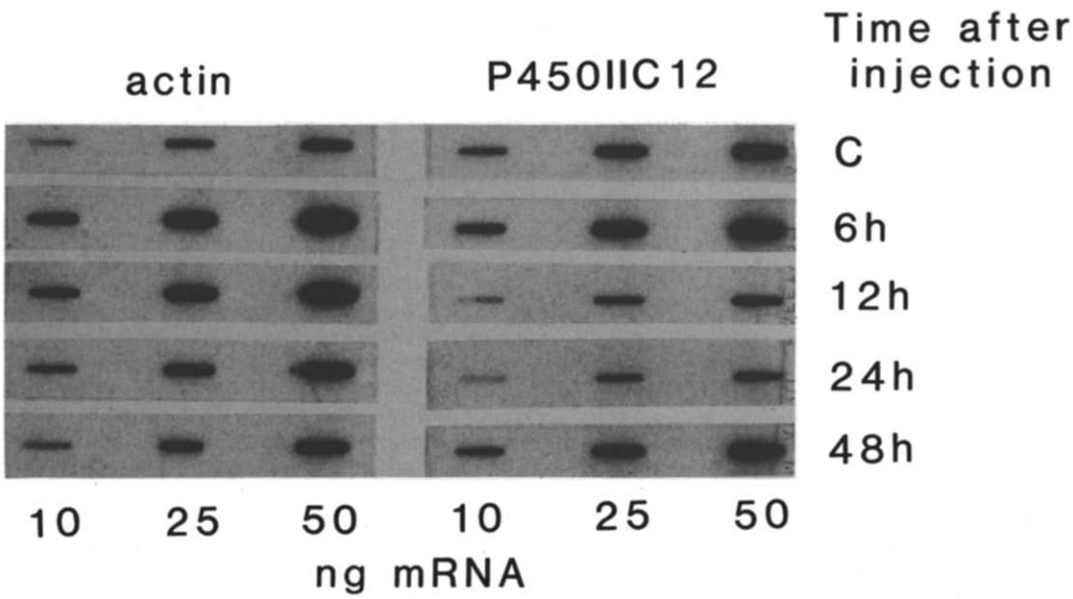

Fig. 2. RNA slot blot showing elevation of actin mRNA and suppression of P450IIC12 mRNA in livers of poly rI·poly rC-treated rats. Rats were treated with poly rI·poly rC (10 mg/kg, i.p.) and killed at the times shown. Hepatic poly $(A)^+$ RNA was prepared and blotted on nylon filters. The amount of RNA was determined by UV absorbance. The filters were probed with β-actin and P450IIC12 cDNA probes, as described in the text, and subjected to autoradiography. Each row represents poly $(A)^+$ RNA from a representative rat liver from each treatment group. Quantitative data summarizing the results for all rats in the experiment, and corrected for poly $(A)^+$ contents of the samples, are shown in Figs. 1 and 4. The figure is a composite of nonadjacent rows from a single filter (for each probe). C, saline-treated controls.

Table 1. Effect of poly rI·poly rC on hepatic microsomal contents of cytochrome P450 and cytochrome b_5 in female rats

Time after injection (hr)	N	Total P450 (nmol/mg protein)	Cytochrome b_5 (nmol/mg protein)
Experiment 1			
Control	5	1.01 ± 0.07	0.92 ± 0.07
6	5	0.92 ± 0.05	0.83 ± 0.01
12	5	0.86 ± 0.03*	0.84 ± 0.09
24	5	0.63 ± 0.04*	0.71 ± 0.04*
48	4	0.72 ± 0.03*	0.71 ± 0.02*
Experiment 2			
Control	5	0.97 ± 0.04	0.90 ± 0.03
24	5	0.50 ± 0.04*	0.56 ± 0.05*
48	5	0.72 ± 0.03*	0.73 ± 0.04*
72	5	0.80 ± 0.07*	0.71 ± 0.05*

Adult female rats were injected with 10 mg/kg poly rI·poly rC and killed at the times indicated. Control rats were killed 24 hr after injection of vehicle. P450 and cytochrome b_5 contents of the microsomes were assayed as described in the text. Values are means ± SEM.

* Significantly different from control value, $P < 0.05$.

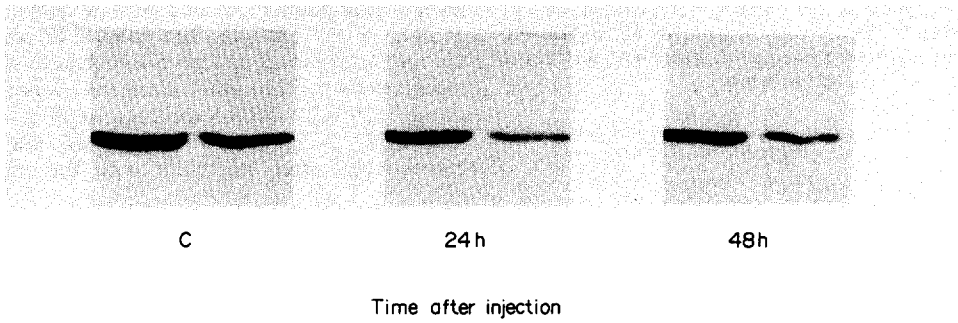

Fig. 3. Western immunoblot showing suppression of microsomal P450IIC12 apoprotein in livers of poly rI·poly rC-treated rats. Rats were treated as described in the legend of Fig. 1. Western blotting of hepatic microsomes was done as described in the text, and the blots were probed with a monoclonal antibody to P450IIC12. Each panel represents blots of 5.0 and 2.5 μg of hepatic microsomes from a single rat liver representative of its treatment group. The panels are nonadjacent lanes taken from a single nitrocellulose filter. C, saline-treated animals.

inducer (Figs. 2 and 4). However, by 48 hr after treatment, P450IIC12 mRNA levels had rebounded to 147% of control levels ($P < 0.05$) and then began declining towards normal by 72 hr (Fig. 4). A small increase in the mean levels of P450IIC12 mRNA 6 hr after injection marginally escaped being statistically significant.

DISCUSSION

This study shows that hepatic expression of the protein and mRNA products of the *CYP2C12* gene are suppressed after treatment of female rats with the interferon inducer poly rI·poly rC. A greater suppression of P450IIC12 mRNA compared to its microsomal apoprotein was observed (29% of control vs 51% at 24 hr, respectively), and from the 12-hr time point onward the kinetics of suppression and recovery of the two gene products indicated that the primary level of control of the apoprotein levels probably resides in modulation of the mRNA levels (Fig. 3). However, 6 hr after poly rI·poly rC treatment, levels of P450IIC12 apoprotein had already begun to decline, whereas those of the mRNA were elevated at this time. Although the lack of statistical significance of either of these effects precludes any definite conclusion, these trends may indicate that there could also be a posttranslational effect of the treatment on turnover of P450IIC12 apoprotein or on protein translation. Gooderham

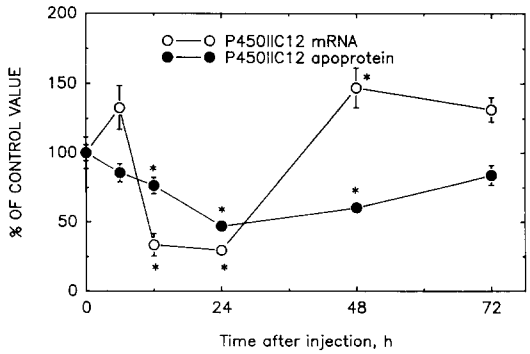

Fig. 4. Time course of suppression of P450IIC12 protein and mRNA by poly rI·poly rC in livers of female rats. Adult female rats were administered poly rI·poly rC as described in the legend of Fig. 1, and were killed at the times indicated. Relative hepatic levels of P450IIC12 apoprotein and mRNA were determined by Western blotting and poly $(A)^+$ RNA slot-blotting, as described in the text. The relative poly $(A)^+$ RNA concentrations of the samples were measured using the oligo-$d(T)_{16}$ probe as described. For P450IIC12 mRNA determinations, the number of animals in each group was the same as in Fig. 2. For P450IIC12 apoprotein measurements, the number of animals in each group was 0 (control), 10; 6 hr, 5; 12 hr, 5; 24 hr, 10; 48 hr, 9; and 72 hr, 4. The figure summarizes data from the animals used in experiments 1 and 2, Table 1. Key: (*) significantly different from control group, $P < 0.05$. The absence of an error bar means that the standard error for a given point is smaller than the size of the symbol.

and Mannering [4] have reported that total poly $(A)^+$ RNA from livers of mice treated with poly rI·poly rC shows a decreased rate of translation in a cell-free system compared to RNA from untreated mice. The same authors also showed earlier that microsomal protein degradation is transiently stimulated after poly rI·poly rC treatment of mice [19].

Although many P450 genes are expressed in the livers of a given animal at a given time, in female rats P450IIC12 is one of a few isozymes that is constitutively expressed at high levels. Based on Western blotting experiments, it can be estimated that P450IIC12 can account for 25–50% of the spectrally measurable P450 in female rat liver microsomes [7]. Suppression of total microsomal P450 and of P450IIC12 by poly rI·poly rC treatment were of similar magnitudes and followed similar time courses (compare Table 1 and Fig. 3). This indicates that although suppression of P450IIC12 must contribute significantly to the decline in total microsomal P450, it can only account for a portion of that decrease. One must conclude either that other P450 isozymes are similarly suppressed, or that increased heme turnover [2] contributes to the decline in spectrally measurable holoenzymes. The previous study from my laboratory on suppression of P450IIC11 in male rat livers by interferon inducers led to a similar conclusion [5]. Pertinent to this discussion, Renton and Knickle [1] have calculated that the increased heme turnover seen after treatment with interferon inducers can account for only about an 8% decrease in total P450. It should be noted also that the effect of poly rI·poly rC in suppressing microsomal proteins is not specific for P450: cytochrome b_5 content was also decreased in both female (this study) and male [5] rat livers.

The experiments presented here do not allow us to conclude that the suppression of P450IIC12 by poly rI·poly rC treatment is a consequence of induction of interferon production. However, it is now well established that pure interferons do suppress hepatic P450 content *in vivo* [1, 6, 18, 20], with a more rapid onset of action than those of interferon inducers [18]. Recombinant interferon-γ suppresses P450IIIA2 protein and mRNA when injected into rats [6]. Moreover, poly rI·poly rC actually raises the concentration of P450 in a hepatocyte culture system [21]. The possible mechanisms whereby interferons may cause suppression of P450 gene expression have been reviewed recently [1]. The demonstrations here and elsewhere that hepatic P450 suppression is accompanied by a decline in the mRNAs for at least four P450 enzymes [1, 5, 6] argue that pretranslational mechanisms are of primary importance. The results are not inconsistent with the hypothesis [1, 18] that induction of 2′,5′-oligoisoadenylate synthase, leading to activation of RNase L, could explain the decreases in P450 mRNAs. In support of such a mechanism, Moochhala and Renton [18] have reported that interferon-mediated suppression of P450 and some of its activities are inhibited by puromycin. However, such data could be explained equally well by an effect on transcription of hepatic P450 genes, mediated by a protein suppressor that is induced by interferons. Indeed, a recent report showed that treatment of mice with poly rI·poly rC actually increases hepatic levels of total poly $(A)^+$ RNA [4]. Clearly, direct studies on the effects of interferons and their inducers on specific P450 gene transcription are indicated.

As discussed earlier, measurement of actin mRNA content is a widely used and accepted method to correct for possible errors generated in RNA blotting experiments caused by inaccuracies in estimation of the RNA contents of samples. The data presented here clearly show that hepatic actin gene expression in female rats is profoundly affected by poly rI·poly rC treatment, indicating that actin mRNA content can only be used as a control if it is established that the experimental conditions do not affect expression of this gene. I confirmed that actin mRNA levels were elevated in the livers of poly rI·poly rC-treated rats (a) by purifying poly $(A)^+$ RNA from total RNA, thus minimizing or eliminating the contribution of contaminants to the absorbances of the samples at 260 nm, and (b) by measuring the relative levels of poly $(A)^+$ RNA in each sample with a $d(T)_{16}$ probe. The apparent increase in actin mRNA content of the liver after poly rI·poly rC treatment was reduced from about 7-fold when total RNA was used (data not shown) to 3.1-fold when mRNA was used. This could reflect an increase in poly $(A)^+$ content of hepatic RNA, as has been described in poly rI·poly rC-treated mice [4]. However, I observed no statistical differences in poly $(A)^+$ RNA

yields from total RNA in control and treated rat livers (data not shown).

In view of the demonstrated effect of poly rI·poly rC treatment on actin gene expression in female rat livers, I have re-analyzed our results from the previous study in which we reported the effects of poly rI·poly rC on P450IIC11 in male rat livers [5]. Mean actin mRNA contents in total hepatic RNA from male rat livers were not affected significantly by poly rI·poly rC treatment (data not shown). Thus, there appears to be a substantial sex difference in the effect of poly rI·poly rC on hepatic actin gene expression. The basis of this sex difference is unclear at the present time.

Renton and Knickle have noted that interferon synthesis is induced by a variety of viral and bacterial infections, as well as by immunomodulators [1], and suggested that interferons may mediate the suppression of hepatic drug metabolism and P450 caused by these agents. Indeed, the pretranslational suppression of P450IIC12 (this study) and P450IIC11 [5] by interferon inducers is comparable to that seen with bacterial endotoxin [13]. However, interleukin-1, an inflammatory mediator that profoundly affects hepatic gene expression, has been shown to decrease murine hepatic microsomal P450 levels and certain drug-metabolizing activities *in vivo* [22, 23]. Contemporary studies in this laboratory have yielded evidence that interleukin-1 [24] can suppress hepatic P450 gene expression in rats. Another cytokine, tumor necrosis factor, can also suppress hepatic P450 content, but this appears to be via stimulation of interleukin-1 release [23]. Thus, the relative contributions of interferons and interleukins to suppression of hepatic P450 gene expression during infectious or inflammatory conditions, and the extent of their interactions, may depend on the specific inflammatory stimulus, as is true for extrahepatic inflammatory responses [25].

The most important physiological regulator of P450IIC12 expression in rat liver is the sexually differentiated growth hormone secretory pattern [7]. At this time, it cannot be excluded that an effect of poly rI·poly rC or the induced interferon on pituitary growth hormone secretion may contribute to the observed suppression of P450IIC12 expression.

In conclusion, this study shows that the suppression of hepatic microsomal P450 content caused by treatment of rats with poly rI·poly rC was accompanied by a decrease in microsomal content of P450IIC12, and that this suppression was achieved, at least in part, by a pretranslational mechanism. Concomitantly with the P450IIC12 suppression, poly rI·poly rC treatment caused an elevation in the levels of hepatic actin mRNA.

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